Abstract

The central nervous system (CNS) responds to injury or damage by activating immune cells to clear dying cells and debris. Previous studies have suggested that there is a correlation between CNS remyelination and macrophage appearance at the site of damage. In macrophages, Activin A is a marker for immunoregulatory response to induce differentiation of precursor cells. Due to this involvement, we hypothesize that activin A is a growth factor that induces differentiation of myelinating cells (oligodendrocytes) in the CNS. This study identified characteristics of indirect signaling between oligodendrocytes and macrophages that regulate remyelination. Supernatants containing secreted factors were harvested from wild type macrophages and *mertk* (a receptor protein responsible for activating Activin A transcription)-deficient macrophages over a time course. Oligodendrocytes were treated with these supernatants for 48 hours and then inspected for morphological changes indicative of differentiation. When comparing the extent of differentiation between the wild-type and knockout group, supernatants from wild-type macrophages produced a greater proportion of cells with higher level differentiation. This suggests that a secreted factor(s) is dependent on the presence of the Mertk pathway is responsible for increased differentiation levels of oligodendrocytes. By understanding what cellular and indirect interactions control remyelination, we can work to further identify cellular therapeutic targets that may inhibit demyelinating diseases.

Introduction

The central nervous system (CNS) is particularly prone to physical and pathological damage because of its limited regenerative capabilities. This damage can manifest in the form of a demyelinating disease, such as multiple sclerosis. As a reaction to damage, the CNS can also produce an immune response to clear the damaged cells and cell debris through the resident macrophages: the microglia. As seen through previous studies, there appears to be a correlation between CNS remyelination and macrophage appearance at the site of damage (Kotter). This study will identify characteristics of a possible signaling between oligodendrocytes, the targets of signaling, and macrophages that may positively regulate remyelination.

Oligodendrocytes are responsible for the myelination of neurons located in the CNS, similar to a single Schwann cell myelinating an axon in the peripheral nervous system (PNS) with the key difference being that unlike Schwann cells, oligodendrocytes are able to myelinate multiple axons from different neurons. Oligodendrocytes produce a myelin sheath by extending multiple processes that then wrap around different axons. This sheath acts as an insulating mechanism that allows electrical impulses to travel down the axon rapidly and efficiently. In patients with demyelinating diseases, these interactions between oligodendrocytes and neurons are broken down. This loss of myelin results in slowed or blocked impulses leading to possible sensory and motor impairment. In adults, damage to neurons is nearly permanent but myelin damage can be repaired through the use of oligodendrocyte precursor cells.

Oligodendrocyte precursor cells (OPCs) are the cell line that are able to differentiate into mature oligodendrocytes. These OPCs are primarily found in the subventricular zone of the adult brain and are activated and migrate when needed (Goldman). After areas of white matter are damaged, these OPCs migrate to the site of demyelination and differentiate into mature oligodendrocytes which will then remyelinate the axons of neurons. OPC differentiation can be tracked through immunohistochemistry (IHC) staining using various late stage-specific antibodies that recognize Oligodendrocyte marker O4 and MBP (myelin basic protein) of maturing OPCs. Another method of tracking differentiation levels in OPCs is by observing morphology changes in the cells. Cells maturing into oligodendrocytes will show a greater extent of bifurcation in their processes, eventually allowing them to myelinate multiple axons. While IHC allows us to determine which cells were differentiated, only by observing morphology change can we determine different extents of differentiation. For all intents and purposes, we will use the latter method in this study.

In the innate immune response, macrophages are the cells responsible for the primary response to foreign microbes in many different tissues and relevant to this study, they recognize apoptotic or dying cells. Not only are they responsible for clearing foreign particles and dying cells at the site of injury, but they also positively regulate the overall immune response. Macrophages use Tyrosine-protein Kinase Mer (Mertk), a cell surface protein that is a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family, to recognize and to signal the phagocytosis of apoptotic cells. Cells such as macrophages, or retinal epithelial cells, that have the mutations in the *mertk* gene are deficient in adequately clear apoptotic cells (Scott and Gal).

A particular protein secreted by macrophages after digestion of foreign microbes or dying cells is Activin A, a signal known to enhance proliferation of microglia and is associated with modulating inflammatory responses to bacterial meningitis (Wilms). Activin A is also a growth factor known to be involved in the differentiation of various other cell types (Kotter). According to unpublished data from the Matsushima lab, cells that are unable to clear apoptotic cells via phagocytosis are unable to produce Activin A. Because of this role in inflammatory response, it may also act as a signal or share the same pathway as the signal responsible for OPC differentiation at damaged sites, demonstrating the communication between macrophages and OPCs. Preliminary data from the Matsushima lab has shown a peak of Activin A to occur at the 24-hour time point. In this study, I postulate that there is cell signaling between macrophages and OPCs via Activin A, positively regulating OPC differentiation into mature oligodendrocytes.

Methods

Mice.

Male and female C57BL/6 (wildtype) and *mertk*$^{-/-}$ mice at least 8 weeks old, were used for peritoneal exudate cell (PEC) extraction. Both strains of mice were bred and maintained in accordance to IACUC-approved protocols at UNC. 72 hours before extraction, the mice were intraperitoneally injected with 3mL of thioglycollate (as in Scott).

Peritoneal Exudate Cells (PEC) Extraction.

Mice were euthanized via isofluorane and cervical dislocation. After sterilization with 70% ethanol, the mice were then injected intraperitoneally with approximately 3 mLs of

versene followed by gentle massaging of the peritoneum. A midsagittal incision was then made in the skin without puncturing the peritoneum so that the two can be separated. Using a Pasteur pipette, a small hole was created and air was inserted into the peritoneal cavity to create a tenting effect. The versene mixture was collected using the pipette and transferred into a 15 mL conical tube. This was centrifuged at 4°C for 5 minutes at 1500 rpm (275 g). The supernatant was then discarded and the pellet was resuspended in 10mL of RPMI (1640) media with 5% fetal bovine serum. After the suspension was diluted to $4x10^5$ cells/mL, 500uL of the suspension was placed in each well of a 24-well plate, thereby transferring $2x10^5$ PECs to each well. PECs were washed 2x prior to use to eliminate non-adherent cells leaving adherent macrophages at approximately 85-95% purity.

Apoptosis Stimulation.

Thymocytes at 5×10^6 were stimulated for apoptosis by introduction of 2uM dexamethasone to the thymocyte media. This was then incubated at 37°C for four hours prior to placement onto macrophage cultures.

Stimulation of PECs by Apoptotic Cells (ACs).

The apoptosis-induced thymocytes (after incubation) were washed three times with phosphate-buffered saline (PBS) and refed with 500uL of RPMI (1640) with 1% fetal bovine serum. These apoptotic thymocytes were added to the PECs in the 24-well plate and incubated at 37°C for 1 hour. The PECs were washed extensively to remove undigested thymocytes and then allowed to incubate up to 24 hrs in serum-free media.

PEC Supernatant Collection.

Supernatants from each well of PECs were collected using disposable pipette tips at 4 different time points: 0hr, 6hr, 12hr, and 24hr. 0 hour supernatants were collected immediately after AC stimulation, 6 hour supernatants were collected 6 hours after AC stimulation, and so on. The collected supernatants were transferred to 1.5 mL Eppendorf tubes and spun down at 2 RCF (4.7×10^3 RPM) for five minutes to remove cellular debris. After spinning, all of the supernatant except for approximately the last 20uL was transferred to a separate tube and then frozen at -20°C. There were macrophages from C57BL/6 wild type control mice and *mertk*$^{-/-}$ mice for supernatant collection at these time points. For a larger sample size, the conditions for each time point were replicated two more times (total of 3 wells of macrophages for each time point), creating a total of 24 samples of supernatant collections for wild type control and *mertk*$^{-/-}$ mice.

Obtaining OPCs.

Oli-neu cells were used as representative cells of OPCs. These cells are oligodendrocytes that were transfected with the *t-neu* oncogene, immortalizing the cell line (Jung). These cells were originally transfected and obtained by the Trotter lab at the University of Heidelberg and have been cultured by the Matsushima lab at UNC. This study uses the E9-G4 clone of Oli-neu cells previously isolated and frozen by the Matsushima lab.

Culturing Oli-neu Cells.

The Oli-neu cells were cultured in T-25 flasks and collected in a 15mL conical tube using versene solution after reaching approximately 80% confluence. These cells were then spun down at 4°C for 10 minutes at 1000 RPMs, washed with PBS twice, and resuspended in Oli-neu media (as prepared in Jung). The Oli-neu cells were diluted to 1×10^6 cells/mL. This suspension

of cells was platted in a 96-well plate with 50uL of resuspended cell solution aliquoted into each well, producing wells with $5x10^4$ cells in each well. This plate of Oli-neu cells was incubated at 37°C for 24 hours.

Stimulation of Oli-neu Differentiation.

50uL of supernatants from each time point were placed into the wells in the 96-well plate containing Oli-neu cells. As a negative control, three wells received RPMI with 5% fetal bovine serum as opposed to macrophage supernatant. The macrophage supernatants were incubated with the Oli-neu cells for 48 hrs to allow for differentiation. Differentiation was assessed by the number of processes from the cell body and the number of branching designated as either primary, secondary, tertiary and multiple branching. A minimum number of at least 100 Oli-neu cells were assessed and quantified in each well. Replicates numbers were averaged and statistical analyses applied.

Statistical analyses.

Graphs with multiple time points were analyzed for significance using two-way ANOVA between supernatants from WT and Mertk deficient macrophages. This was done by quantification of Oli-neu cells grouped based on primary, secondary, tertiary, complex, or no differentiation. A separate statistical analysis was done for significance using an unpaired t-test on tertiary differentiation between 24 hour wild-type and *mertk*$^{-/-}$ supernatants.

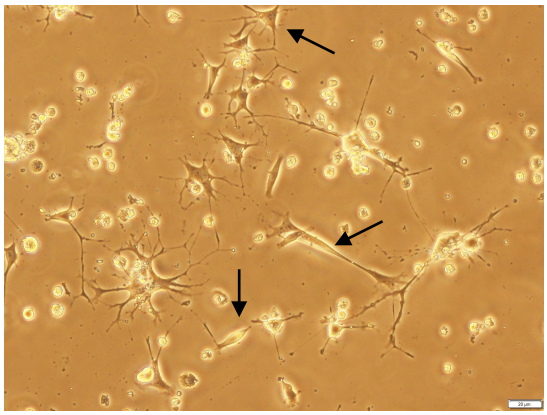
Proteomic Assay

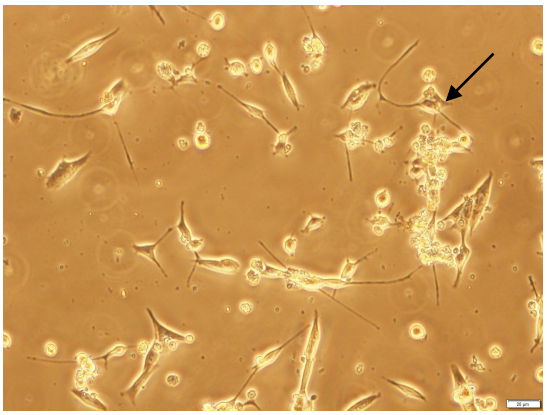
Wild-type and *mertk*$^{-/-}$ macrophage supernatants were run for protemic profling through the UNC Proteomics Core Facility. Proteins in the supernatant samples went through a trypsin digest and were then analyzed using mass spectrometry. This raw data was then run

with a reference sample for the MASCOT and processed with the Proteome Discoverer software. Identified proteins that were expressed at higher concentrations in wild-type macrophage supernatants as opposed to mertk$^{-/-}$ macrophage supernatants were run through the DAVID program, sponsored by NIH, and categorized by known function.

Results

A time course of supernatants harvested from macrophage-stimulated cultures was conducted to determine the optimal time point in which factors that induced oligodendrocyte differentiation were secreted. Representative pictures of the three wells in the 96-well plate were taken to quantify the extent of processes produced by Oli-neu cells. Cells were labeled as having either primary, secondary, tertiary, or complex differentiation based on the extent to which processes from the cells had bifurcated. Cells that extend processes without branching are labeled as having primary differentiation. Secondary differentiated cells have bifurcation in at least 1 process. Tertiary differentiation is characterized by a second branching point on the same process and complex differentiation has 3 or more branching points.

A. 

B. 

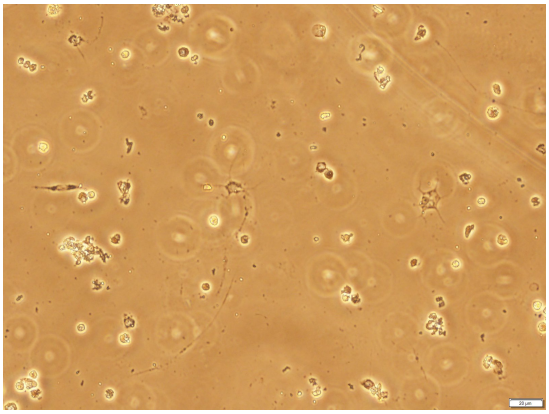
C. 

Figure 1: Oli-neu differentiation after 48 hours of incubation with arrows pointing at cells with tertiary differentiation. The white boxes represent a length of 20 microns. A. Addition of 24-hour supernatants from wild-type macrophage exposed to apoptotic cells. B. Addition of 24-hour supernatants from *mertk*$^{-/-}$ macrophage exposed to apoptotic cells. C. Addition of macrophage media (control) consisting of only unconditioned RPMI with 5% fetal bovine serum.

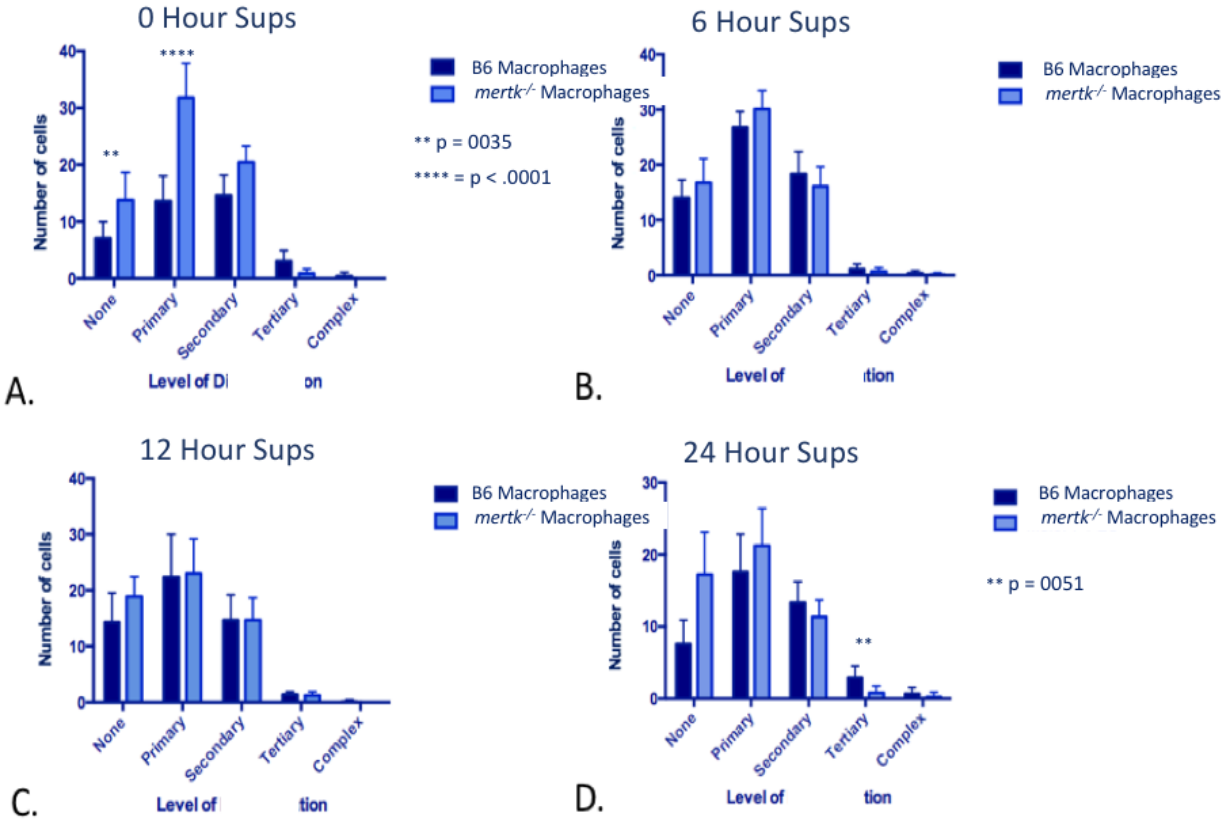


Figure 2: Graphical representation of oli-neu cells grouped by differentiation levels for supernatants from each time point. A. Assay with 0 hour supernatants. B. Assay with 6 hour supernatants. C. Assay with 12 hour supernatants. D. Assay with 24 hour supernatants. Statistical analysis of differentiation using 2-way ANOVA produced a p-value less than .0001, demonstrating that the difference between wild-type and *mertk*$^{-/-}$ is significant. Separate analysis of only the tertiary morphology with 24 hour supernatants using an unpaired t-test produced a p-value of .0051. This data is representative of three independent experiments.

The control group, Oli-neu cells that received the macrophage media alone without exposure to apoptotic cells, had little differentiation with the majority of differentiation being classified with primary processes. On the other hand, the wild-type group and *mertk*$^{-/-}$ group both showed greater levels of differentiation. When comparing the extent of differentiation between the wild-type and knockout group, the wild-type macrophage supernatant produced a greater amount of secondary, tertiary, and complex differentiation whereas the *mertk*$^{-/-}$ macrophage supernatant did not allow for as much higher level differentiation, particularly the

tertiary morphology. The 24-hour time point for supernatant produced the most significant differences between the wild-type and *mertk*$^{-/-}$ while other earlier time points show a similar, yet not as suggestive, correlation. Additional statistical analysis of the number of oligodendrocytes undergoing tertiary conformation showed supernatants from wild type macrophages significantly increased branching compared to *mertk*$^{-/-}$ supernatants (p=0.0051 unpaired t-test).

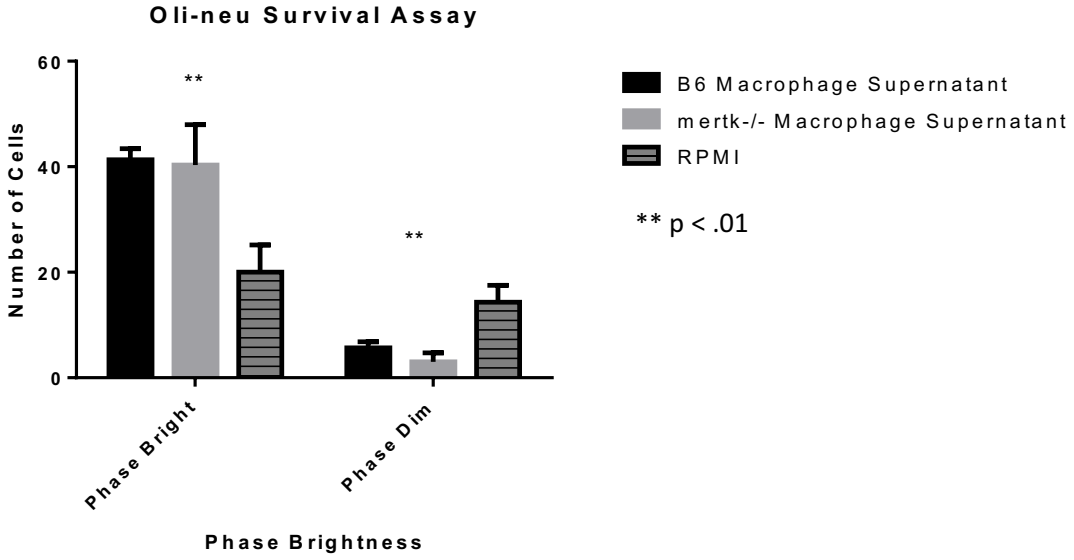


Figure 3: Graphical representation of Oli-neu cell survival assay. Phase bright cells are representative of viable cells and phase dim cells are representative of dead cells. Statistical analysis using a two-tailed t-test produced a p value less than .01, demonstrating a significant difference between cells stimulated with macrophage supernatants and cells stimulated with only unconditioned RPMI. No significant difference was found between cells stimulated with B6 macrophage supernatants and cells stimulated with *mertk*$^{-/-}$ macrophage supernatants.

Comparison of cells stimulated with macrophage supernatant with cells stimulated with control media (unconditioned RPMI with 5% FBS) produced another interesting result with regards to viability. Supernatant from macrophages exposed to apoptotic cells appeared to support Oli-neu cells where serum components were omitted (Figure 1A and B). The phase

contrast bright cells are an indication of viability and intact membrane. In contrast, supernatants from macrophages not exposed to apoptotic cells that were in the absence of serum supplement, did not support Oli-neu cells after 48 hrs as shown in phase contrast dim cells (Figure 1C). Here, the majority of OPCs in the control wells were dead. After quantification of these cells, no significant differences were observed between cells stimulated with wild-type and *mertk*$^{-/-}$ macrophage supernatants. Though, significant differences were observed between cells stimulated with macrophage supernatants as opposed to unconditioned RPMI. Cells stimulated with macrophage supernatants demonstrate higher levels of viable cells and lower levels of dead cells. This suggests that factors secreted from macrophages exposed to apoptotic cells provide nutritional support of Oli-neu cells. There data are consistent with the notion that macrophages may be providing OPCs with extracellular factors that induce activation, growth, and differentiation.

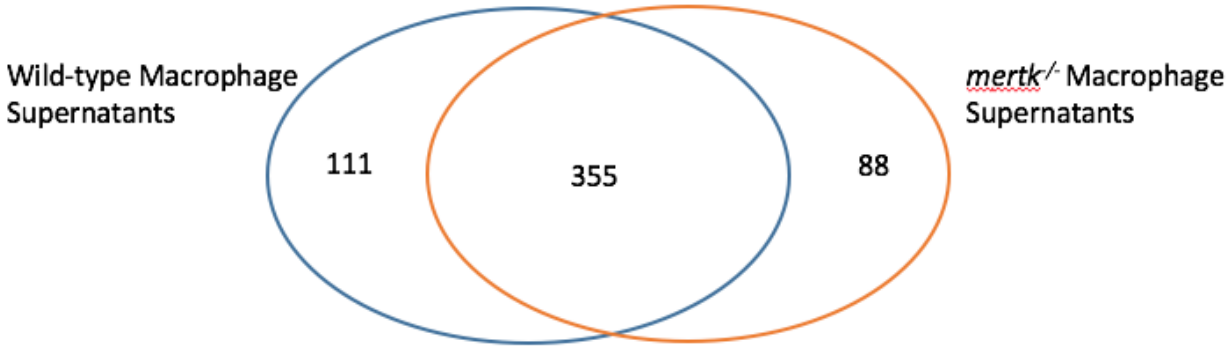


Figure 4: This diagram demonstrates the number of proteins differentially expressed in wild-type and *mertk*$^{-/-}$ macrophage supernatants. Proteins expressed by wild-type macrophages at two fold or greater concentrations as compared to mertk$^{-/-}$ macrophages were placed in the wild-type macrophage supernatant category. The same was done for proteins expressed at two fold or greater concentrations by the mertk$^{-/-}$ macrophages. Those proteins that were expressed at similar levels were placed in the overlapping area.

Proteomic analysis of supernatants showed that 111 proteins were expressed at a two-fold or greater concentration in wild-type macrophage supernatants and 88 proteins were

expressed at a two-fold or greater concentrations in *mertk*$^{-/-}$ macrophage supernatants. 355 proteins were found to be expressed in similar concentration between the two types of supernatants. As wild-type macrophages induce higher levels of differentiation of OPCs, the differentiation factor is assumed to be found in the 111 proteins expressed at higher levels from wild-type macrophages.

Discussion

This study aimed to find evidence for cell signaling between macrophages and OPCs. Activin A is a growth factor that is a part of the TGF superfamily and is known to support proliferation of various undifferentiated stem cells (Natale). Along with macrophages, it has also been associated with acute CNS tissue injury and inflammation (Wilms) thereby making it an ideal macrophage target signal for remyelination studies as neuroinflammation is a symptom of many demyelinating diseases. Results show that supernatants from macrophages in WT mice stimulated with apoptotic thymocytes increase OPC differentiation and allow for cell survival, suggesting that a secreted signal from macrophages can influence OPCs.

Supernatants from wild-type macrophages (*mertk*$^{+/+}$) produced a greater proportion of cells with higher level differentiation, which suggests that a signal going through the Mertk pathway is responsible for increased differentiation in OPCs. This signal must be one that is secreted by the macrophages, rather than a cell membrane component, because the supernatant was spun in a tabletop centrifuge forcing any cells or cell debris to the bottom of the tube, preventing transfer to the subsequent tube. Therefore, instead of direct contact between macrophages and OPCs through intermembrane proteins, the signal is produced by a

secreted factor remaining in the supernatant. Not only is this signal secreted, but this study also suggests that there is a higher quantity of this signal in 24-hour supernatants as opposed to 0, 6, and 12-hour supernatants.

One potential secreted factor that is known to induce differentiation in several cell types is Activin A. Previous unpublished data from the Matsushima lab has suggested that Activin A may be a component of the Mertk pathway, reaching peak secretion 24 hours after stimulation with apoptotic cells. These characteristics of Activin A align with observed results from this experiment, supporting the hypothesis that Activin A is positively regulating differentiation of OPCs. Although these experiments do not prove that Activin A is solely responsible for OPC differentiation, additional experiments could be conducted to determine whether Activin A is present in these cultures and functions to induce OPC differentiation, so as to determine cause and effect. We attempted to conduct such experiments but they were difficult to execute due to reduced robustness of our Oli-neu differentiation assay. Furthermore, *mertk*$^{-/-}$ macrophages should not be able to clear apoptotic cells by phagocytosis and therefore would not produce Activin A to induce differentiation. However, some Oli-neu cells treated with *mertk*$^{-/-}$ macrophage supernatants were able to obtain higher levels of differentiation, suggesting the cell line changed properties or that other factors may be contributing to OPC maturation as well.

Additionally, supernatants from both wild-type and *mertk*$^{-/-}$ mice allowed for increased survival of OPCs. This suggests that both supernatants contained a survival factor that allows OPCs to better adjust to stressful environments. *Mertk*$^{-/-}$ supernatants seem to produce OPCs will similar survival levels to wild-type supernatants, indicating that the unknown survival factor

in question is not regulated through the Mertk pathway. Similar to the differentiating factor mentioned above, this signal seems to be one that is secreted as it was carried in the macrophage supernatants.

It was not surprising to see primary differentiation within the control group that received no supernatants, as the cell line was immortalized during a stage in which the OPCs had a bipolar morphology. This bipolar morphology is consistent with the control results and its presence in other experimental wells. However, in this experiment and a previous experiment not shown, there were greater numbers of Oli-neu cells with tertiary morphology exposed to supernatants from wild type macrophages suggesting that remyelination may be partly dependent on the presence of myeloid cells. It remains to be seen whether microglia replicate a similar function in generating factors that promotes OPC differentiation. This is an important concept to pursue with further experimentation because histopathology of lesions from MS brains suggest there is a lack of differentiation of OPCs.

Acknowledgement

First and foremost, I would like to thank my thesis advisor and Principal Investigator, Dr. Glenn Matsushima. Not only did he teach me the skills needed for these experiments, but he also advised me throughout the process whenever trouble with research or writing. When I had trouble, he led me towards the next logical step while allowing the work to be my own. I would also like to thank Dr. Laura Herring for her assistance in compiling the proteomics data and her advice on how to extrapolate the most relevant data. Additionally, I would like to thank Dr. Amy Maddox and my fellow Biology Honors Thesis students for helping to form both

my written thesis and my presentation. Lastly, I would like to thank Dr. Blaire Steinwand for sponsoring my research project.

References

Gal, A., Li, Y., et al. (2000). Mutations in MERTK, the Human Orthologue of the RCS Rat Retinal Dystrophy Gene, Cause Retinitis Pigmentosa. *Nature genetics*, ***26***(3), 270-271. DOI:10.1038/81555

Goldman, Steven A. and Sim Fraser. (2005), Neural Progenitor Cells of the Adult Brain. *Stem cells: nuclear reprogramming and therapeutic applications (Novartis Foundation Symposium 265).* 66-97.

Jung, M., et al. (1995), Lines of Murine Oligodendroglial Precursor Cells Immortalized by an Activated neu Tyrosine Kinase Show Distinct Degrees of Interaction with Axons In Vitro and In Vivo. *European Journal of Neuroscience,* **7**, 1245-1265. DOI: 10.1111/j.1460-9568.1995.tb01115.x

Kotter, M. R., et al. (2001), Macrophage Depletion Impairs Oligodendrocyte Remyelination Following Lysolecithin-Induced Demyelination. *Glia*, **35**(3), 204–212. DOI: 10.1002/glia.1085

Natale, David R.C., et al. (2009). Activin Promotes Differntiation of Cultured Mouse Trophoblast Stem Cells Towards a Labyrinth Cell Fate. *Developmental Biology,* **335**(1), 120-131. DOI:10.1016/j.ydbio.2009.08.022

Scott, Rona S., et al. (2001), Phagocytosis and Clearance of Apoptotic Cells is Mediated by MER. *Nature,* **411**, 207-211.

Wilms, H., et al. (2010), Regulation of Activin A Synthesis in Microglial Cells: Pathophysiological Implications for Bacterial Meningitis. *Journal of Neuroscience Research,* **88**(1), 16-23. DOI: 10.1002/jnr.22185